Junctional Epidermolysis Bullosa Keratinocytes in Culture Display Adhesive, Structural, and Functional Abnormalities

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An unusual, elongated, refractile cell morphology was observed in keratinocytes cultured from three patients with non-lethalis forms of junctional epidermolysis bullosa (JEB). To determine whether these changes might be related to altered cell adhesion, keratinocyte strains established from one patient were examined for adhesive, structural, and functional characteristics. JEB keratinocytes expressed keratin tonofilaments, as determined by staining with AE1 monoclonal antibodies and direct observation of tonofilaments by electron microscopy. JEB keratinocytes showed diminished cell-substratum adhesions, judged by interference reflection microscopy. Areas of diminished cell-substratum adhesion corresponded to F-actin–rich cell adhesions (focal adhesions) and not to cellular areas that abundantly express hemidesmosomal antigens. Analysis of cell-substratum adhesion by electron microscopy revealed extensive areas of cell-substratum separation in JEB keratinocytes that were not present in normal keratinocytes maintained in serum-free medium. Normal keratinocytes displayed numerous regions of focal contact between the ventral plasma membrane and the culture substratum, but these structures were not seen in JEB keratinocytes. Bundled actin filaments (stress fibers) were greatly diminished in expected regions of cell-substratum adhesion in JEB keratinocytes and, instead, displayed disorganized individual filaments. The growth rate of JEB keratinocytes was quite slow in culture, with a population doubling time of 2.7 d versus 1.5 d for normal keratinocytes under identical conditions. JEB keratinocytes also displayed a reduced ability to aggregate into colonies upon exposure to medium with increased extracellular calcium. JEB keratinocytes thus display adhesive, structural, and functional abnormalities that suggest this cell type may be central to the pathogenesis of junctional epidermolysis bullosa. Study of affected keratinocytes could be important to characterize associated molecular pathologies. *J Invest Dermatol 97:849–861, 1991*

Epidermolysis bullosa (EB) is a heterogenous group of inherited disorders characterized by formation of blisters at sites of friction or minimal trauma [1]. In EB simplex, the level of split is within the basal keratinocyte, whereas splitting occurs within the lamina lucida in junctional EB and below the lamina densa in dystrophic EB. Within each major group, differences in clinical features have led to further subcategorization. For example, within the group of junctional epidermolysis bullosa (JEB), six subtypes are recognized, their severity ranging from death in infancy to survival into adulthood [1,2].

Specific subtypes of epidermolysis bullosa may be caused by one or more genetically encoded defects in cell adhesion molecules or structures that mediate epidermal-dermal cohesion. Some cases of JEB, especially the more severe lethalis type, are characterized by absence or defective formation of the hemidesmosome-anchoring filament complex [3] or of associated antigens [2,4–6]. Other cases, however, are not associated with defective hemidesmosomes [3], and some lamina lucida-associated antigens that are absent in JEB are absent also in dystrophic EB [4,5]. This suggests that two possible basal keratinocyte-basement membrane adhesion mechanisms might be operative in JEB.

Adhesion of cultured cells to culture surfaces, analogous to cell-basement membrane adhesion, is produced by two structures termed *focal adhesions* and *close contacts* that mediate tight and intermediate adhesion, respectively, of cells to extracellular matrix or tissue culture surfaces [7,8]. Focal adhesions, which are frequently membrane insertion points for actin-containing stress fibers, are morphologically and functionally distinct from hemidesmosome dense plaques, which are membrane insertion points for intermediate (keratin-containing) filaments [7–13]. Focal adhesions and close contacts can be directly visualized by interference reflection microscopy, which analyzes cell-substratum adhesion points, or by electron microscopy [8,10,11]. The biochemical composition of focal adhesions includes F-actin, α-actinin, vinculin, talin, and α/β integrin molecules [7,9–11,13–16], which are distinct from identified molecular components of hemidesmosomes [12]. Integrin-containing focal adhesions have been shown to mediate strong adhesion of keratinocytes in culture to laminin and other extracellular matrix molecules [10,11]. Because laminin is a component of the skin basement membrane, and laminin-specific integrin subtypes are localized in the basal laminal membrane of the epidermis, this type of adhesion-substratum adhesion could be highly relevant to epider-

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Abbreviations:
BMZ: basement membrane zone
EB: epidermolysis bullosa
JEB: junctional epidermolysis bullosa

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mal basement membrane cohesion in normal or pathologic skin conditions.

In this study we report that cultured JEB keratinocytes show striking morphologic, adhesive, and growth differences when compared to cultured normal keratinocytes. JEB keratinocytes showed diminished focal adhesions and altered actin cytoskeleton compared to normal keratinocytes. These data provide evidence that some JEB subtypes may be caused by a specific keratinocyte adhesive defect and suggest that cultured JEB keratinocytes could be used to further characterize cellular or molecular features of this disease.

MATERIALS AND METHODS

Junctional Epidermolysis Bullosa Keratinocytes were cultured from epidermis of three patients with junctional epidermolysis bullosa: males ranging in age from 9 to 19 years. Keratinocytes cultured from each showed an unusual elongated or spindle morphology. The 19 year old was hospitalized for treatment of widespread chronic erosions by keratinocyte grafting and thus provided a source of repeated cultured keratinocytes for grafting and laboratory studies. Multiple keratinocyte cultures, all of which showed similarly aberrant cellular features, were used for the studies described in this manuscript. In general, first-passage keratinocytes (secondary cultures) were used for characterization. Some clinical features of this patient have been previously published [17]. He displayed moderately extensive bullous lesions, but was categorized into the nonletalis group based on age of survival. Electron microscopic examination of his bullous disorder showed a split within the lamina lucida region of the basement membrane zone with retention of numerous hemidesmosomes in basal keratinocytes, similar to other described cases of JEB [3]. All biopsy material was procured after obtaining informed consent and IRB approval.

Keratinocyte Culture Normal human keratinocytes were cultured from neonatal foreskin after dispase separation of epidermal sheets using growth factor–supplemented MCDB 153 medium (KGM, Clonetics Corp, San Diego, CA) as previously described [18]. Keratinocytes from JEB patients were cultured from epidermal sheets obtained from suction blisters [17] or roofs of newly formed spontaneous bullae. For routine culture and growth measurement, keratinocytes were cultured on plastic tissue culture surfaces. Photomicrographs of cells in culture were taken using a 10× objective on an inverted Nikon microscope. For immunofluorescence and interference reflection microscopy, keratinocytes were cultured on untreated glass coverslips sterilized by gamma irradiation.

Immunofluorescence Microscopy and Interference-Reflection Microscopy To visualize cellular proteins by immunofluorescence microscopy, cells were fixed with PBS-buffered 4% formaldehyde. Cells were permeabilized by incubation for 5 min with 0.5% Triton-X100 in PBS. Cytokeratins were detected with an equal AE1 monoclonal anti-keratin antibodies (gift of T.-T. Sun, NY University, New York). Well characterized bullous pemphigoid antibodies that recognize 180-kD and 230-kD hemidesmosomal proteins [19,20] were a generous gift from Dr. Luis Diaz (Medical College of Wisconsin). A second bullous pemphigoid serum was obtained from Dr. J.-C. Bystryn (New York University School of Medicine, New York). Rabbit antibodies to desmoplakins I/II [21] were a generous gift from Dr. James Nelson (Fox Chase Cancer Center). FITC-conjugated, $F(ab')_2$ fragments of goat–anti-mouse antibodies (Tago, Burlingame, CA) were used to visualize binding of mouse primary antibodies. FITC-conjugated phalloidin (Molecular Probes, Junction City, OR) was reacted directly with permeabilized cells. Photomicrographs were taken with a Zeiss photomicroscope using epifluorescence illumination at an original magnification of 630×. Interference-reflection microscopy was performed on formalin-fixed cells with and without Triton-X100 detergent permeabilization, as previously described [8] using a Zeiss 63× antireflex objective.

Electron Microscopy Normal or JEB keratinocytes in primary culture were fixed directly in tissue culture flasks with warm MCDB 153 medium (Hepes-buffered) containing 2–2.5% glutaraldehyde for a minimum time of 1 h. Cells were postfixed with OsO_4 while attached to tissue culture plastic and were stained in situ with uranyl acetate. While still attached to tissue culture plastic, cells were dehydrated and embedded in Epon 812 as previously described [22]. Silver sections of embedded cells were cut parallel and perpendicular to the tissue culture surface on a Reichert-Jung Ultracut E ultramicrotome. Sections were re-stained with uranyl acetate and lead citrate prior to viewing with a JEOL 100 CX electron microscope.

Growth Measurements To determine the rate of proliferation of normal or JEB keratinocytes, 10^5 keratinocytes obtained from trypsinized, washed secondary cultures were seeded in 25-cm^2 tissue culture flasks (Corning #25100) in KGM. The number of cells attached after 1 d of incubation was determined by trypsinization, neutralization of trypsin with medium containing 10% bovine serum, and cell counting with a Coulter counter. Growth of cells after 3, 7, and 10 d was also determined by direct cell counting. All measurements were performed on duplicate flasks, and results are expressed as the mean ± standard deviation.

RESULTS

Altered Morphology of Junctional Epidermolysis Bullosa Keratinocytes We observed that keratinocytes cultured from three patients with junctional epidermolysis bullosa (JEB) showed an unusual elongated, spindle-form morphology compared to keratinocytes cultured from normal controls. Figure 1*A* shows the morphology of primary normal keratinocytes in serum-free, growth factor–supplemented MCDB 153 medium. Normal keratinocytes cultured from more than 500 samples of normal skin (neonatal and adult) prepared from dispase-separated epidermal sheets or suction blisters have shown growth of small, epithelioid cells in colonies typical of that illustrated in Fig 1*A*. In contrast, an altered appearance of keratinocytes from three patients with JEB was consistently observed. Keratinocytes from each of these individuals displayed an elongated, spindle-form shape with enlarged cytoplasm typical of the primary colony illustrated in Fig 1*B*. The morphologic differences between normal and JEB keratinocytes were maintained on subsequent subculture of primary cultures (Figs 1*C* and *D*, respectively). First-passage JEB keratinocytes showed both elongated, spindle-form cells and less elongated, large epithelioid-type cells. Morphologic differences between normal and JEB cells were best appreciated in cultures in which cells grew into small colonies, but distinct morphologic differences were seen even in cultures soon after cell attachment and spreading. Further characterization of differences between normal and JEB keratinocytes was performed on cells cultured from one of these patients; more than 20 primary keratinocyte cultures initiated from this patient over 12 months of investigation showed consistent morphologic differences compared with normal keratinocytes.

JEB Keratinocytes Express AE1-Reactive Keratin Filaments To confirm that the JEB spindle-form cells expressed cytokeratins typical of epithelial cells, normal keratinocytes and JEB cells were compared for expression of cytokeratins detected by AE1 monoclonal anti-keratin antibodies using immunofluorescence microscopy. Both normal keratinocytes (Fig 2*A*) and JEB cells (Fig 2*B*) showed intense staining with AE1 antibodies, but cultured human dermal fibroblasts showed no reaction with this antibody (Fig 2*C*). Normal keratinocytes and JEB keratinocytes showed abundant keratin-type tonofilaments on electron microscopic evaluation (see below), independently confirming the immunofluorescence findings. To determine whether the altered appearance of JEB keratinocytes might be related to altered cell-substratum adhesion (analogous to cell-basement membrane adhesion in vivo), or to altered expression of another cytoskeletal system, JEB keratinocytes were examined directly for cell-substratum adhesions by interference re-

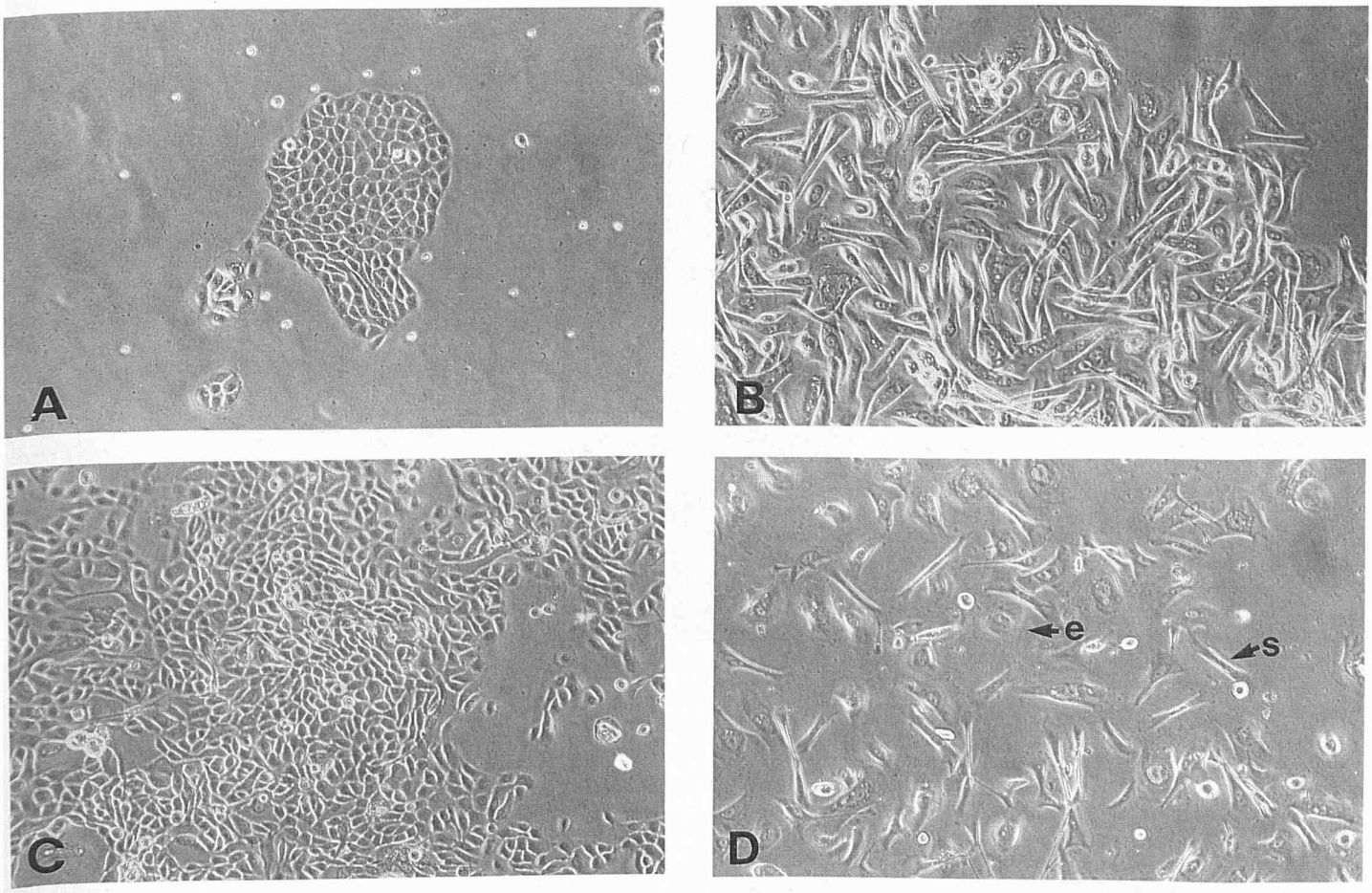

Figure 1. Keratinocytes from junctional epidermolysis bullosa (JEB) show altered morphology. *(A)* Single colony of normal human keratinocytes in a primary culture shows a compact, typical epitheloid morphology. *(B)* A colony of JEB keratinocytes in primary culture shows enlarged, spindle-form keratinocytes. Ruffling membranes are evident in cells at edge of colony. *(C)* Typical appearance of normal human keratinocytes after first subculture. *(D)* Morphologic alteration of JEB keratinocytes is maintained after subculture of primary cells. Both large epitheloid (e) and spindle-form (s) keratinocytes are evident in this secondary culture. Phase contrast micrographs ×80.

flection microscopy and for a polymerized actin cytoskeleton using immunofluorescence microscopy.

Examination of Cell-Substratum Adhesions by Interference-Reflection Microscopy Direct examination of keratinocyte-substratum adhesion to a glass culture surface was performed on formalin-fixed cells using interference reflection microscopy to visualize adhesion structures. It was not possible to examine cells cultured on usual plastic surfaces due to the thickness of tissue culture plastic and a short focal distance of the antireflex objective used for this type of microscopy. Although distinct morphologic differences between normal and JEB keratinocytes were maintained on glass surfaces (borosilicate coverslips), JEB keratinocytes were less elongated than cells maintained on plastic surfaces.

Figure 3*A* shows a typical interference-reflection image of isolated, fixed normal human keratinocytes that have not been treated with detergents or other membrane-disrupting agents. Areas of cell-substratum adhesion appear black or gray, whereas areas of cell-substratum separation appear white in these images. Numerous dark-appearing focal adhesions are arranged in a spoke-like radial pattern at the cell periphery. Some focal adhesions are linear or filamentous along the ventral cell surface, probably corresponding to the position of actin stress fibers in these cells [10,11]. Diffuse, gray-toned areas of close contact are also evident in these normal cells (Fig 3*A*).

The interference-reflection image of JEB keratinocytes is greatly different from normal keratinocytes (Fig 3*B,C*). The cell periphery shows a nearly continuous thin white band of cell-substratum separation that is devoid of clear-cut focal adhesions. The peripheral white band suggests a relatively continuous area of cell-substratum separation at the cell periphery, unlike normal keratinocytes, in which focal adhesions are largely present at this location. In JEB cells, other areas of cytoplasm adjacent to the lateral edge showed white images, suggesting additional areas of cell-substratum separation (best seen in Fig 3*B*). The remainder of the ventral cell surface shows a diffuse, relatively homogeneous gray appearance with the gray-black adhesions in a central, presumably subnuclear location (Fig 3*B,C*). These interference reflection images, which are representative of differences between normal and JEB keratinocytes, suggest a loss of peripheral focal adhesions in JEB keratinocytes with attendant areas of cell-substratum separation. Loss of cell-substratum adhesions in JEB keratinocytes was confirmed by electron microscopic examination of cells grown on usual tissue culture plastic surfaces (see below).

Disorganized Actin-Containing Cytoskeleton in JEB Keratinocytes Because focal adhesions contain f-actin, in addition to other proteins, the cytoskeleton composed of polymerized or filamentous actin was examined by immunofluorescence microscopy using FITC-conjugated phalloidin, which binds to f-actin but not g-actin [23]. Normal keratinocytes showed f-actin organized in a few stress fibers in the cytoplasm and in a number of small radial

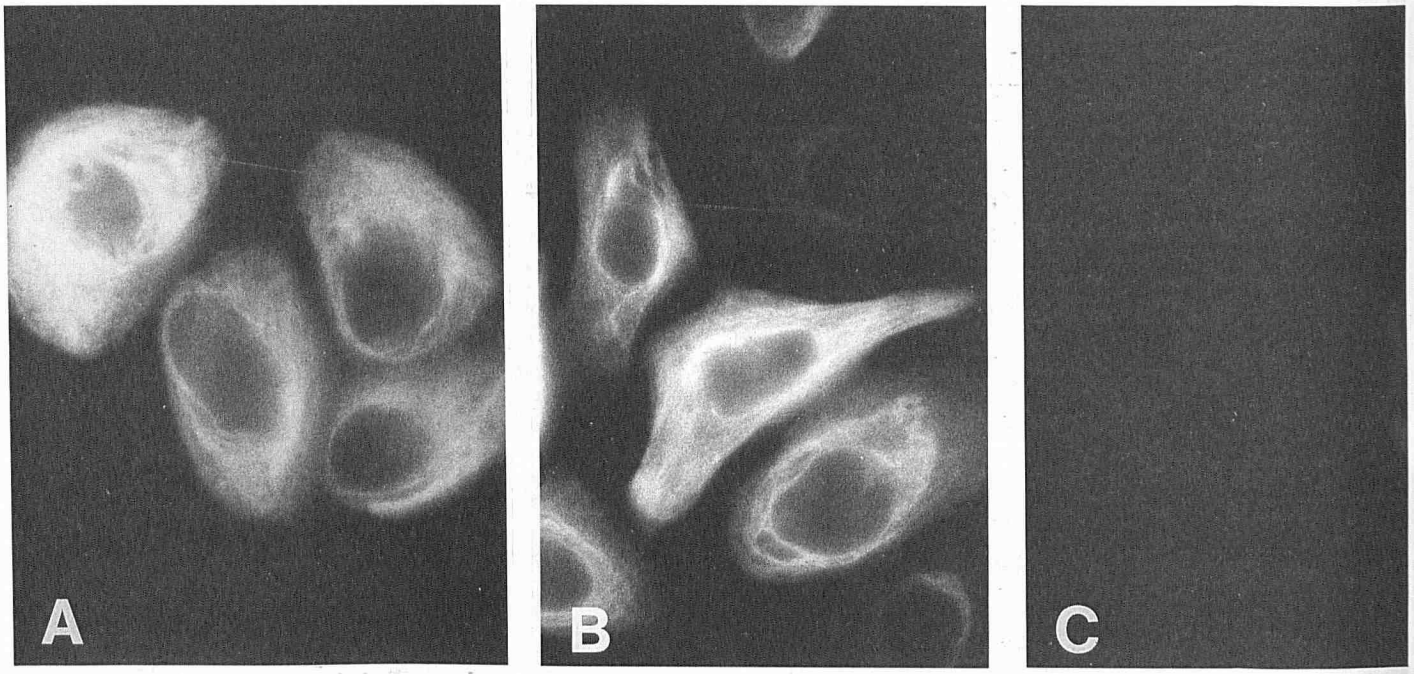

Figure 2. Identification of keratin filaments in cultured cells by immunofluorescence microscopy. AE1 monoclonal antibodies were reacted with normal keratinocytes *(A)*, JEB keratinocytes *(B)*, or a confluent culture of human dermal fibroblasts *(C)*. All photomicrographs ×1800.

spikes at the cell periphery (Fig 4*A,B*). Radial actin spikes appear in the same focal plane and cellular location as peripheral focal adhesions visualized by interference reflection microscopy. Based on these and prior observations [10,11], radial actin spikes visualized by FITC-phalloidin fluorescence appear to correspond to focal adhesions in human keratinocytes. Actin-containing stress fibers visualized in cortical or central cytoplasmic areas may also form focal adhesions, judged from their similar appearance on interference reflection microscopy (Fig 3*A*) and immunofluorescence microscopy (Fig 4*A,B*).

Compared to normal keratinocytes, the f-actin containing cytoskeleton is significantly reduced in JEB keratinocytes (Fig 4*C,D*). Only a few poorly formed radial actin spikes were seen in JEB keratinocytes (*arrowheads,* 4*C,D*). Also, fewer actin-containing stress fibers were seen in cortical and intracellular sites in JEB keratinocytes. A disorganized actin-containing cytoskeleton in JEB kerati-

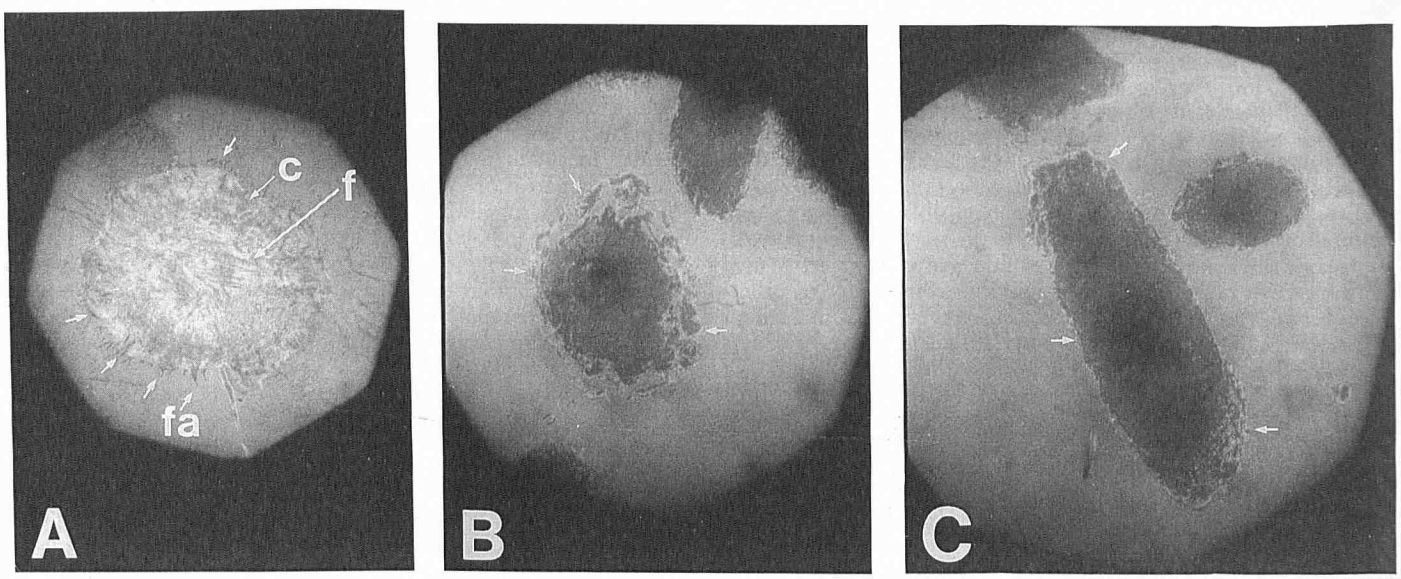

Figure 3. Cell-substratum adhesions viewed by interference reflection microscopy in normal and JEB keratinocytes. *(A)* Normal keratinocytes show focal adhesions (fa) in a radial pattern at the cell edge. Linear or filament-like adhesions (f) are located in the mid-cytoplasmic area. Gray-toned patches of close contact (c) are also evident. *(B,C)* JEB keratinocytes lack peripheral focal adhesions *(small arrows)*, but have extensive gray-colored contact regions located diffusely over the ventral cell surface. Note peripheral white areas of cell-substratum separation in JEB keratinocytes. All photomicrographs ×1512.

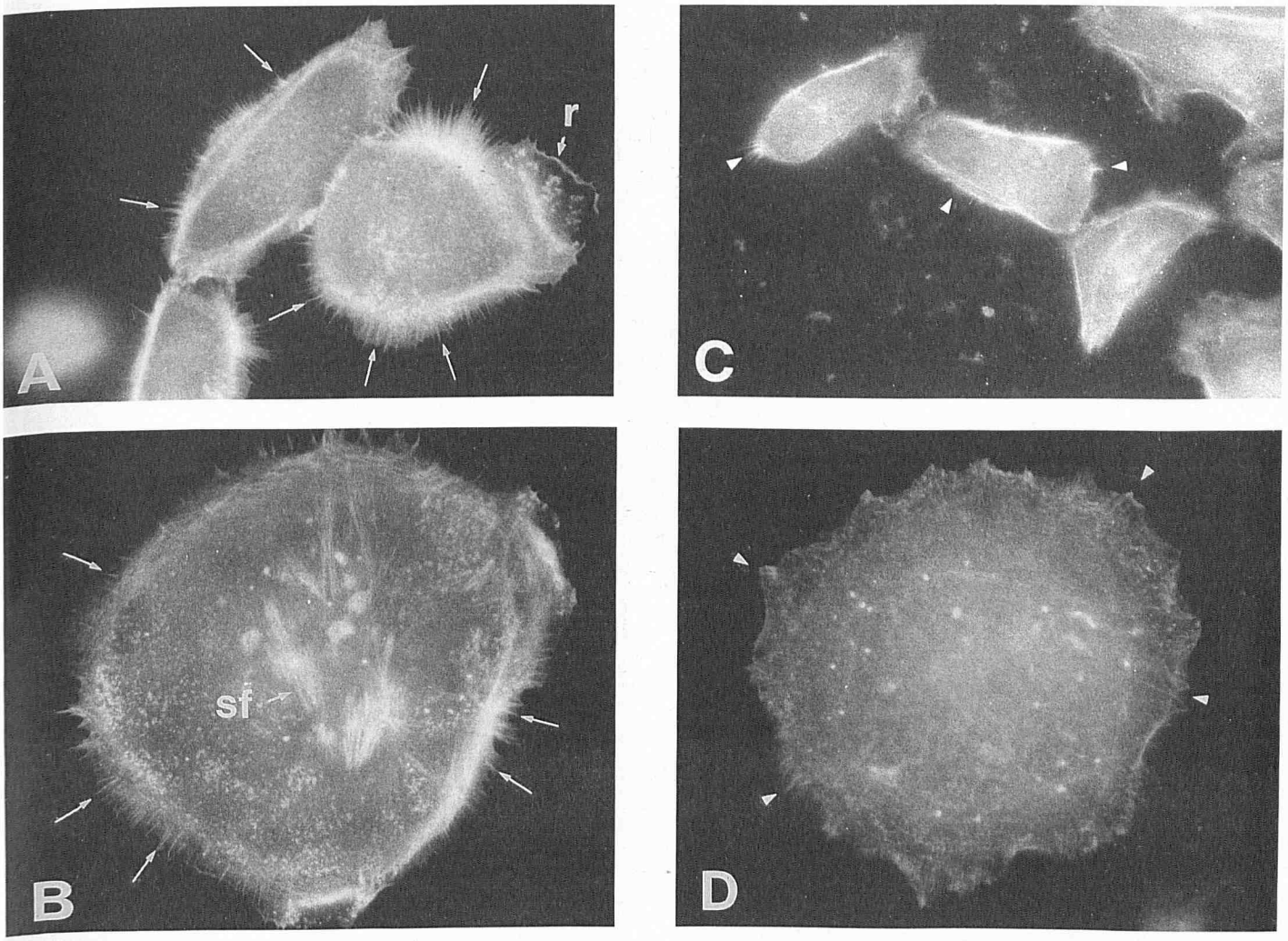

Figure 4. Analysis of polymerized actin cytoskeleton in normal and JEB keratinocytes by immunofluorescence microscopy. Normal keratinocytes *(A,B)* or JEB keratinocytes *(C,D)* were reacted with FITC-phalloidin and were viewed and photographed by epifluorescence illumination. *Arrows* in *A* and *B* indicate typical actin spikes arranged in a radial pattern at the cell periphery. Actin-containing stress fibers are arranged in cortical areas adjacent to the spikes, and some stress fibers are evident in central areas of the cytoplasm, "sf" in *B*. Actin associated with ruffling cell membranes, "r," is shown in *A*. In *(C,D)* arrowheads show small, underdeveloped radial actin spikes. Cortical and internal actin-containing stress fibers are also reduced in *(C,D)*. All micrographs ×1800.

nocytes was confirmed by electron microscopic studies described below.

Expression of Hemidesmosome Antigens by Normal and JEB Keratinocytes Because fewer hemidesmosomes are found in many cases of JEB, we examined normal and JEB keratinocytes for expression of 180-kD and 230-kD hemidesmosome proteins using well characterized bullous pemphigoid antibodies [19,20]. In both normal (Fig 5*A*) and JEB keratinocytes (Fig 5*B*) these antigens were concentrated in a central, perinuclear cytoplasmic location, with only faint fluorescence at peripheral cytoplasmic areas that correspond to radial actin spikes/focal adhesions. Hemidesmosome antigen expression in JEB keratinocytes was not reduced compared to normal keratinocytes, and often fluorescence appeared more intense in JEB cells. A second bullous pemphigoid antiserum gave results identical to those shown in Fig 5*A,B*.

To confirm the impression that hemidesmosome antigens were not concentrated in areas of peripheral cell-substratum adhesion, normal keratinocytes were reacted with bullous pemphigoid antibodies and were viewed sequentially by immunofluorescence and interference reflection microscopy with an anti-reflex objective. Figure 5*C–F* show paired immunofluorescence/interference reflection micrographs. Hemidesmosomal antigens are concentrated in central cytoplasmic-perinuclear areas, whereas dark-appearing cell-substratum adhesions are located peripheral to regions of intense hemidesmosome antigen expression. It should be noted that interference reflection images obtained on cells that have been detergent permeabilized for immunofluorescence microscopy show less precise detail of cell-substratum adhesion points than images shown in Fig 3. Even so, there is a clear discordance between peripheral cell-substratum adhesion points and overall expression of hemidesmosomal proteins. This suggests that peripheral areas of cell-substratum adhesion are mediated chiefly by actin-containing focal adhesions, whereas more central areas of cell-substratum adhesion (diffuse, gray-appearing images on interference-reflection micrographs in Fig 3) might be mediated by other adhesive structures, such as hemidesmosomes. As described below, occasional immature hemidesmosomes were observed in both normal and JEB keratinocytes connecting the ventral cell surface with culture substratum; hemidesmosomes were not located at the cell periphery, but instead were concentrated in sub-nuclear or perinuclear cytoplasmic locations, corresponding to the concentration of hemidesmosomal antigens observed by immunofluorescence microscopy.

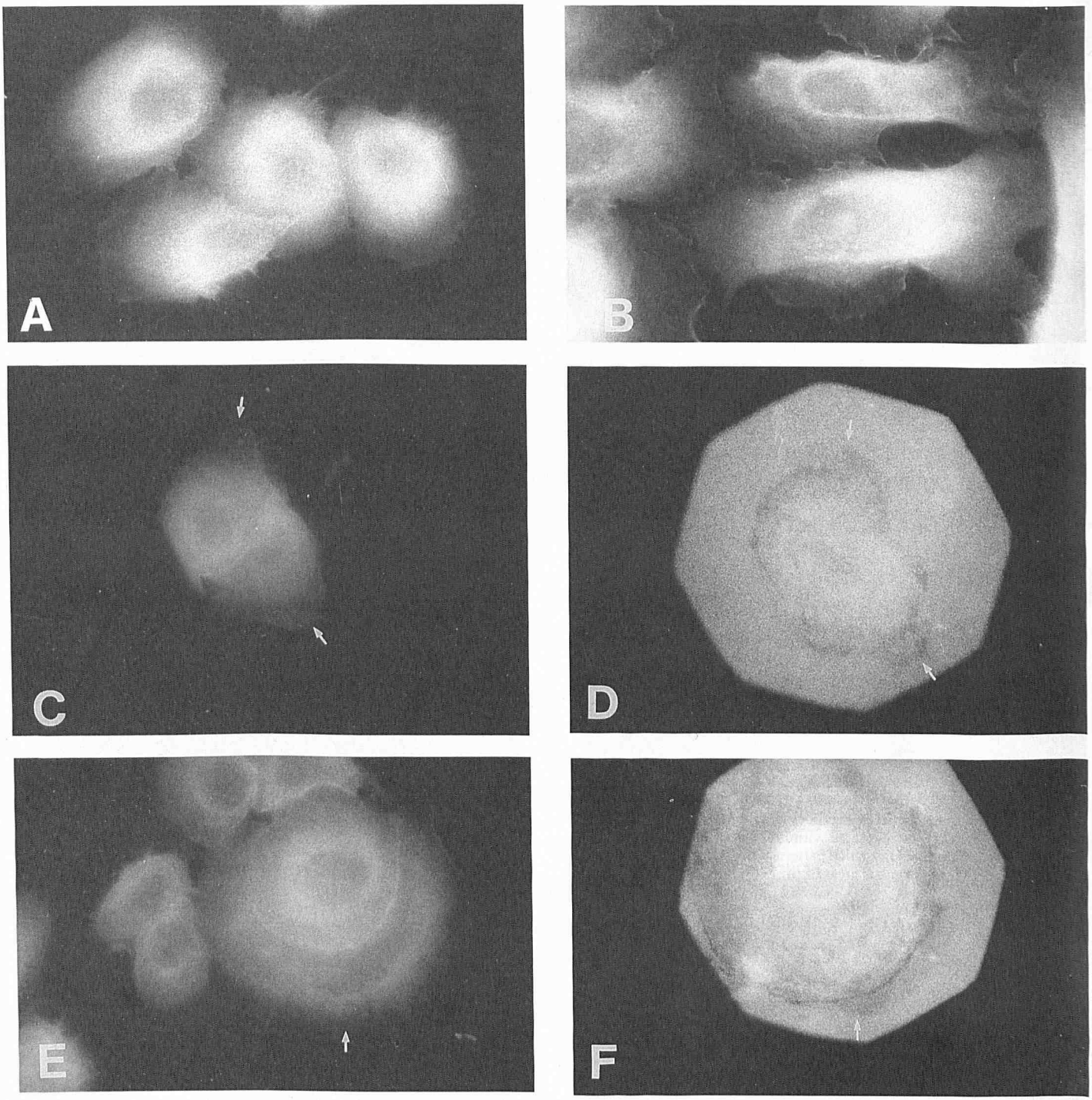

Figure 5. Expression of hemidesmosome (bullous pemphigoid) antigens in normal and JEB keratinocytes. Normal *(A,C,E)* or JEB *(B)* keratinocytes were reacted with bullous pemphigoid antibodies and were viewed by epifluorescence illumination with an antireflex objective. In *D* and *F* the same cell pairs were viewed by bright-light interference-reflection microscopy with the same antireflex objective. *Arrows* in *C–D* and *E–F* mark black-appearing focal adhesions and identical regions of the cells when viewed by fluorescence microscopy. All micrographs ×1800.

Examination of Ultrastructure and Cell-Substratum Adhesion by Electron Microscopy Electron microscopy was used to examine cell-substratum adhesions in normal and JEB keratinocytes at high resolution. Thin sections of cultured cells were prepared perpendicular to the culture surface to best visualize the region between the ventral plasma membrane and the plastic tissue culture surface (cell-substratum adhesions). Figures 6, 7, and 8 show progressively higher-magnification micrographs of morphology, ultrastructure, and cell-substratum adhesions in normal or JEB keratinocytes prepared in this fashion. Other keratinocytes were sectioned parallel to the culture surface to best visualize cytoskeleton filament structure. Figures 9 and 10 show details of radial actin spikes/focal adhesions and cellular cytoskeletons in normal and JEB keratinocytes prepared in parallel sections.

Figure 6*A* shows a low-magnification micrograph of a cross-section through a normal human keratinocyte. This cell contacts the plastic tissue culture surface relatively uniformly over the entire ventral cell surface. In comparison, low-magnification micrographs

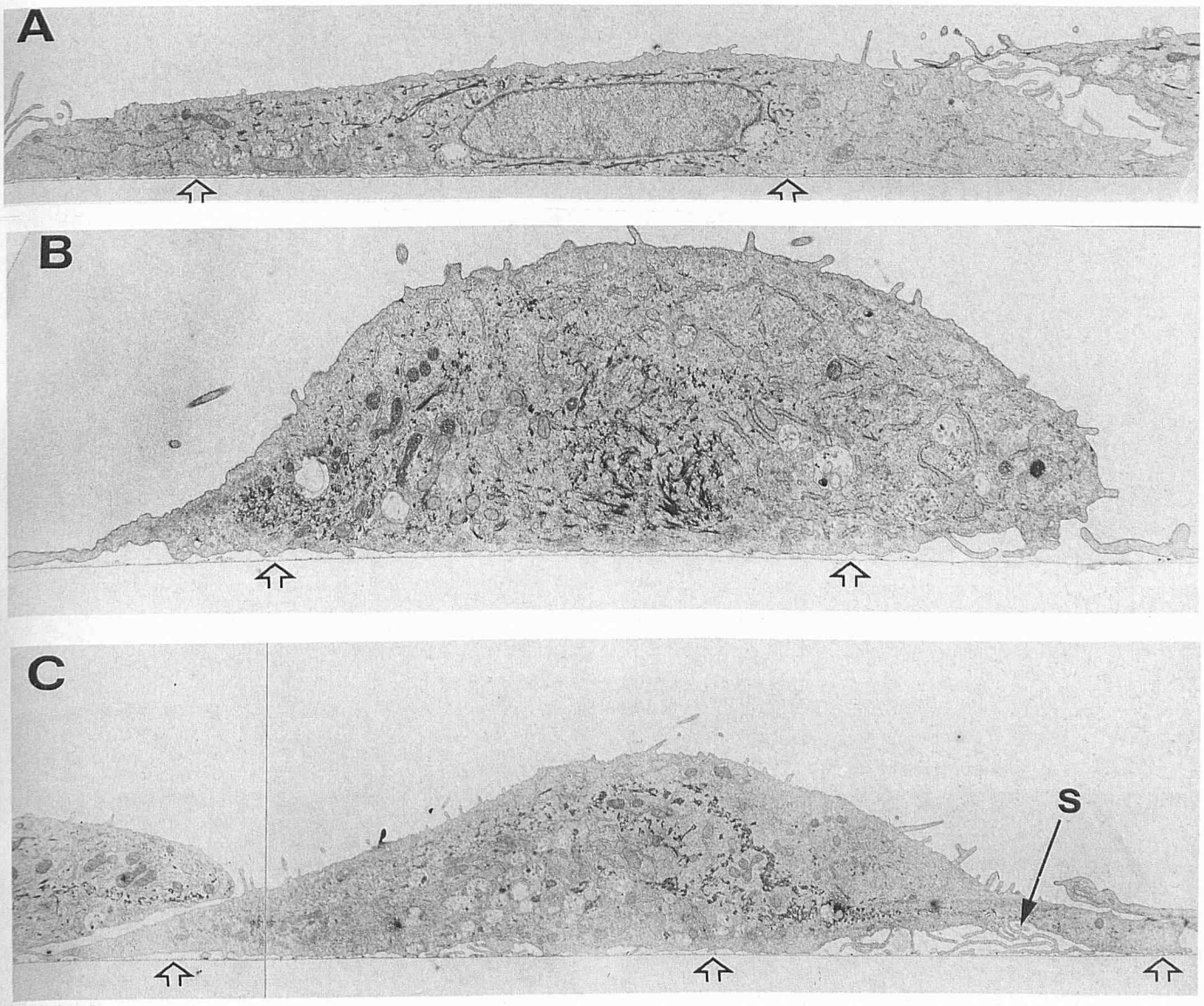

Figure 6. Analysis of cell-substratum adhesion in normal and JEB keratinocytes by electron microscopy. *(A)* Cross-section of normal keratinocyte. Region of cell-substratum attachment is marked by *open arrows. (B,C)* Cross-sections of JEB keratinocytes. In *B* and *C* note extensive areas of separation of ventral cell surface plasma membrane from cell culture surface, indicated by open arrows. All photomicrographs ×4500.

of sections of JEB keratinocytes are shown in Fig 6*B,C*. Both cells show wide areas of separation of the ventral cell membrane from the plastic tissue culture surface near the cell margin, with numerous, thin lamellipodia in these areas of cell-substratum separation. In Fig 6*C* cell-substratum separations are present throughout the entire ventral cell surface, but are widest near the lateral cell edge. The area of widest cell-substratum separation near the lateral edge would account for the band-like white image of cell-substratum separation seen in interference reflection images of these cells.

Higher magnification views of normal and JEB keratinocytes are shown in Figs 7 and 8. Details of cytoplasmic ultrastructure of normal keratinocyte are seen best in Fig 7*A*, and may be compared with those of a JEB keratinocyte shown in Fig 8*A*. The ventral plasma membrane of normal keratinocytes shows close apposition with the culture surface (*open arrows,* Fig 7*A*). In normal cells, the ventral plasma membrane surface appears to directly contact the tissue culture surface in focal regions (Fig 7*B*). These regions of cell-substratum contact have been labeled focal adhesions, because focal adhesions in fibroblasts show a similar degree of ventral plasma membrane-substratum closeness. Other regions of the ventral plasma membrane show a cytoplasmic plaque that is contiguous with fine extracellular filaments and a sub-basal dense plate (Fig 7*C*). These cell-substratum contact points are structurally similar to immature hemidesmosomes seen in early embryonic skin [24] or in basal keratinocytes in wounds that are forming new hemidesmosomes [25,26]. Hemidesmosome cell-substratum adhesions thus have wider areas of separation between the ventral plasma membrane surface and the substratum compared to focal adhesions, which lack structural features of the hemidesmosome. Hemidesmosomes were located principally in central and subnuclear areas of the cell, corresponding to the perinuclear and central cytoplasmic location of tonofilaments (see below).

Cytoplasmic ultrastructure of a typical JEB keratinocyte is shown in Fig 8*A*. Numerous tonofilaments are present in these cells and are concentrated in perinuclear and central cytoplasmic areas. More phagolysosomes were consistently seen in JEB keratinocytes compared to normal cells. However, compared to normal keratinocytes, the most striking difference between JEB and normal cells is in the region of ventral plasma membrane-cell substratum contact. Focal adhesions, or areas of direct cell membrane-culture surface contact,

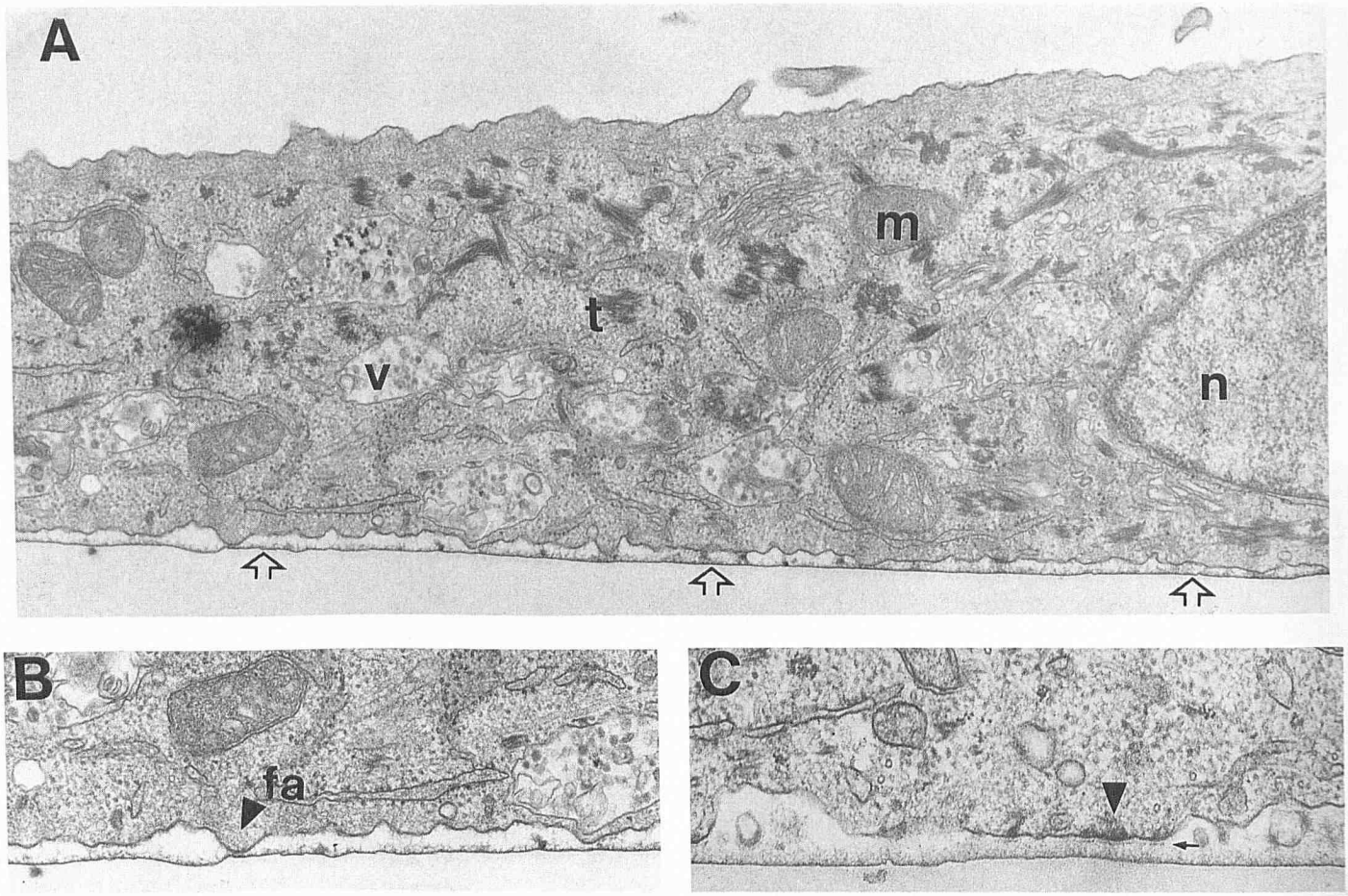

Figure 7. Analysis of cell-substratum adhesion in normal keratinocytes by electron microscopy. *(A)* Normal keratinocyte, showing nucleus (n), mitochrondria (m), tonofilaments (t), vacuoles (v), and area of cell-substratum adhesion *(open arrows)* (×20,000). *(B)* Higher magnification view of cell above, showing focal adhesion "fa" between ventral plasma membrane and cell substratum (×38,000). *(C)* Immature hemidesmosomes form at some regions of cell-substratum contact. Cytoplasmic dense plaque *(arrowhead)* and sub-basal dense plate *(arrow)* that connect to the substratum via fine filaments are characteristic features (×40,000).

were not detected in numerous sections through JEB keratinocytes (Fig 8*A*–*C*). Numerous immature hemidesmosomes, appearing similar to those in normal keratinocytes, were seen in JEB keratinocytes (Fig 8*B*). Frequently, numerous lamellipodia were found separating the ventral plasma membrane surface from the culture substratum (Fig 8*C*). No normal keratinocytes examined showed lamellipodia at the ventral cell surface. Thus, with the exception of hemidesmosome cell-substratum contact points, JEB keratinocytes display poor cell-substratum adhesions and wide areas of cell-substratum separation compared to normal keratinocytes examined under identical culture conditions.

To better examine actin-containing peripheral adhesion spikes and features of cytoskeletal structure, sections of normal and JEB keratinocytes were prepared parallel to the culture surface, and initial sections (those closest to the culture surface) were examined by electron microscopy (Figs 9 and 10).

Figure 9*A* shows cytoplasmic details of a normal keratinocyte in parallel section. Tonofilaments are concentrated adjacent to the nucleus, whereas bundles of actin filaments (stress fibers) are present principally in the peripheral cytoplasm. Near the peripheral actin filaments, a lateral spike protrudes from the plasma membrane (Fig 9*A, arrow*). At higher magnification, this lateral spike is seen to contain numerous, parallel actin filaments that terminate near the end of the process (Fig 9*B*). Enlarged detail of another lateral spike (Fig 9*C*) clearly shows that these lateral spikes are termination points for actin-containing stress fibers. The cytoplasmic area of JEB keratinocytes, which is shown in Fig 10*A*, has marked differences compared to normal cells. In most cells, more numerous tonofilaments are seen in perinuclear and central cytoplasmic areas compared to normal cells. Numerous actin-containing filaments are present peripheral to the tonofilaments, but they are present in a haphazard, unorganized array without bundling or formation of stress fibers. Peripheral plasma membrane areas contain lateral spikes and shorter, more bleb-like protrusions (Fig 10*A, arrows*). Higher magnification of these peripheral spikes (Fig 10*B*) shows that they are largely devoid of well-formed actin filaments, unlike those associated with normal cells that are packed with parallel actin filaments.

These images, which are representative of many cells examined, suggest that actin-containing radial spikes/focal adhesions are very poorly formed in JEB keratinocytes compared to their normal counterparts. The electron micrographs thus confirm immunofluorescence findings with FITC-phalloidin binding that suggest poorly formed peripheral actin spikes and reduced formation of actin-containing stress fibers compared to normal keratinocytes. Electron micrographic observation of tonofilaments confined to perinuclear/central cytoplasmic areas also supports the concentration of hemidesmosomes antigens in these locations, because tonofilaments would be expected to terminate in hemidesmosomes in the ventral cell surface.

Reduced Growth Rate of JEB Keratinocytes Attachment efficiency and proliferation rates of JEB keratinocytes were measured in comparison to normal keratinocytes. In the experiment shown in

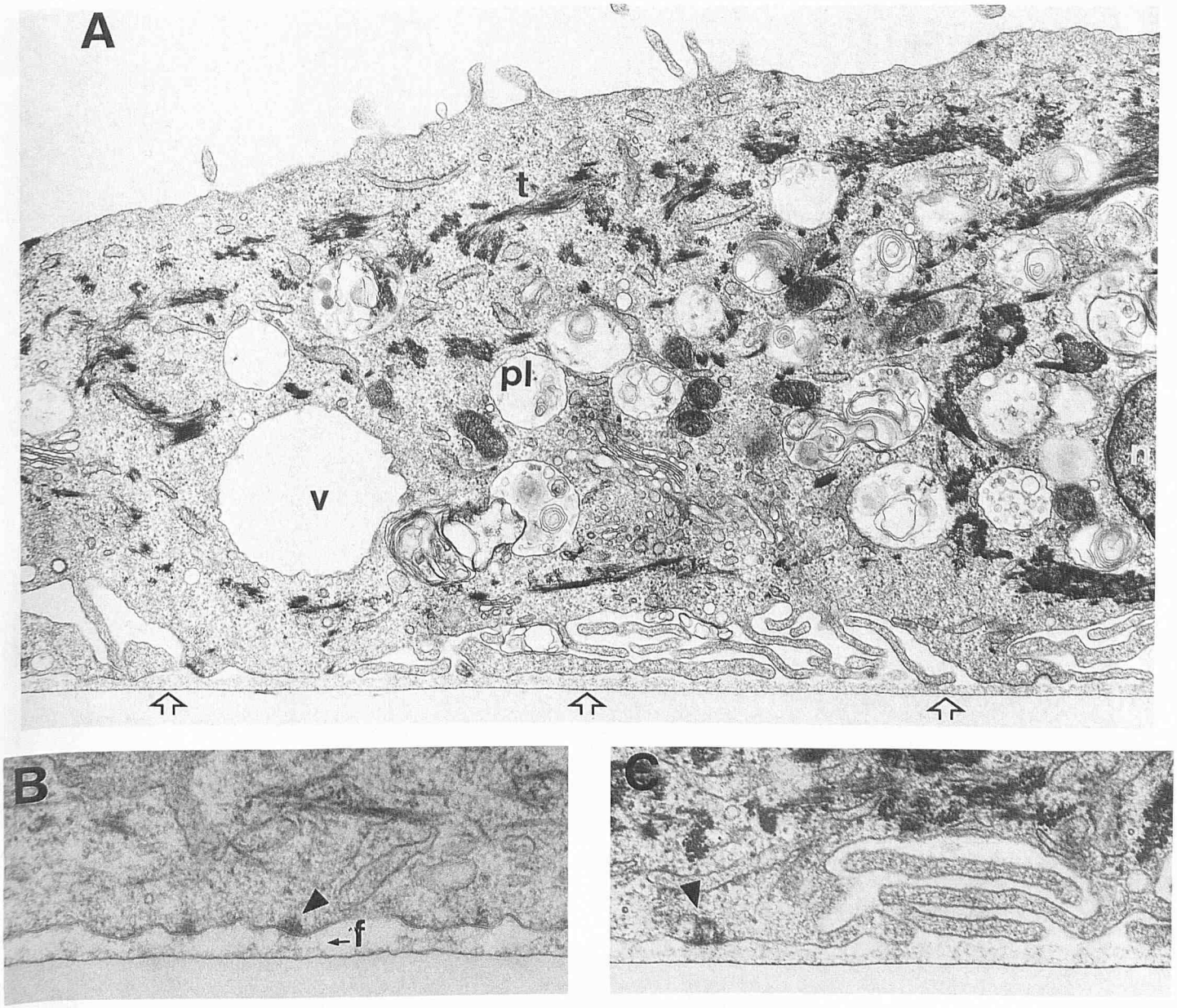

Figure 8. Cytoplasmic detail and area of cell-substratum adhesion in a JEB keratinocyte. *(A)* Tonofilaments (t), vacuoles (v), and numerous phagolysomes (pl) are present in the cytoplasm, adjacent to the nucleus (n) (×20,000). The ventral plasma membrane is separated from the cell-substratum by numerous lamellipodia *(open arrows)*. Focal cell-substratum adhesions were not identified (×20,000). *(B)* Immature hemidesmosome showing cytoplasmic dense plaque *(arrowhead)*, a sub-basal dense plate, and filaments (f) connecting with the cell substratum (×40,000). *(C)* Higher-magnification view of lamellipodia at the ventral cell surface, adjacent to a poorly formed hemidesmosome *(arrowhead)* (×40,000).

Fig 11, equal numbers of JEB keratinocytes or normal keratinocytes were seeded in tissue culture flasks. Attachment efficiency was computed after 1 d of incubation and growth rates were assessed after 3, 7, and 10 d. Initial attachment of JEB keratinocytes was 69% for JEB keratinocytes versus 82% for normal keratinocytes under identical conditions. The growth rate of JEB keratinocytes was reduced compared to normal cells. In the experiment shown in Fig 6, JEB keratinocytes doubled in 2.7 d, whereas normal keratinocytes doubled in 1.5 d. In other growth measurement experiments, JEB keratinocytes failed to grow significantly beyond the first subculture of primary cells.

Reduced Colony Formation of JEB Keratinocytes in High-Calcium Medium Normal keratinocytes shifted to medium containing increased extracellular calcium (1.15 mM) form intercellular desmosomes and grow as colonies or sheets of cells. The ability of JEB keratinocytes to establish desmosomes and form colonies or sheets of cells in high-calcium medium was assessed. The appearance of normal or JEB keratinocytes incubated for 6 h in high calcium (1.15 mM) medium is shown in Fig 12. Normal keratinocytes show progressive intercellular association and eventually form large colonies with few, if any, single (non-paired) cells present. In contrast, JEB keratinocytes form small colonies with a large number of single cells remaining (Fig 12*B*). After incubation for 24 h in high-calcium medium, numerous individual JEB keratinocytes are seen in cultures, whereas none are seen in control cultures (not shown). Analysis of desmosome formation by immunofluorescence microscopy with anti-desmoplakin antibodies revealed that intercellular desmosomes were formed in the small colonies of JEB keratinocytes and in normal keratinocytes (not shown). Thus the dimin-

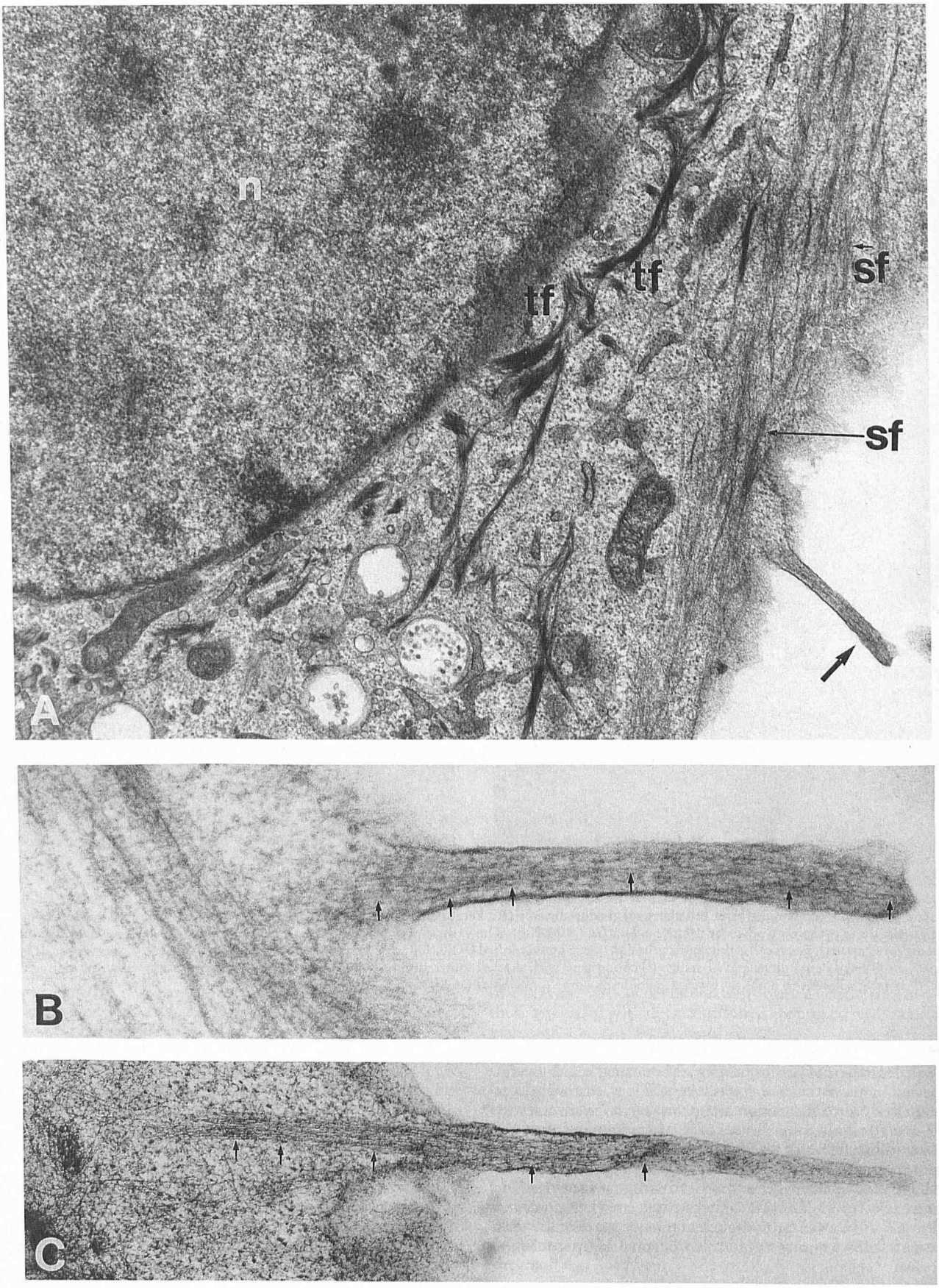

Figure 9. Normal keratinocyte showing details of cytoskeleton and lateral spikes. *(A)* Detail of nucleus (n) and cytoplasm that contains tonofilaments (tf) and bundled actin filaments or stress fibers (sf). A lateral membrane spike is indicated by arrow (×20,800). *(B)* Higher-magnification view of same lateral membrane spike, showing numerous parallel actin filaments throughout length (×63,000). *(C)* Another lateral membrane spike that serves as a termination point for a bundled actin filaments, indicated by *arrow* (×42,500).

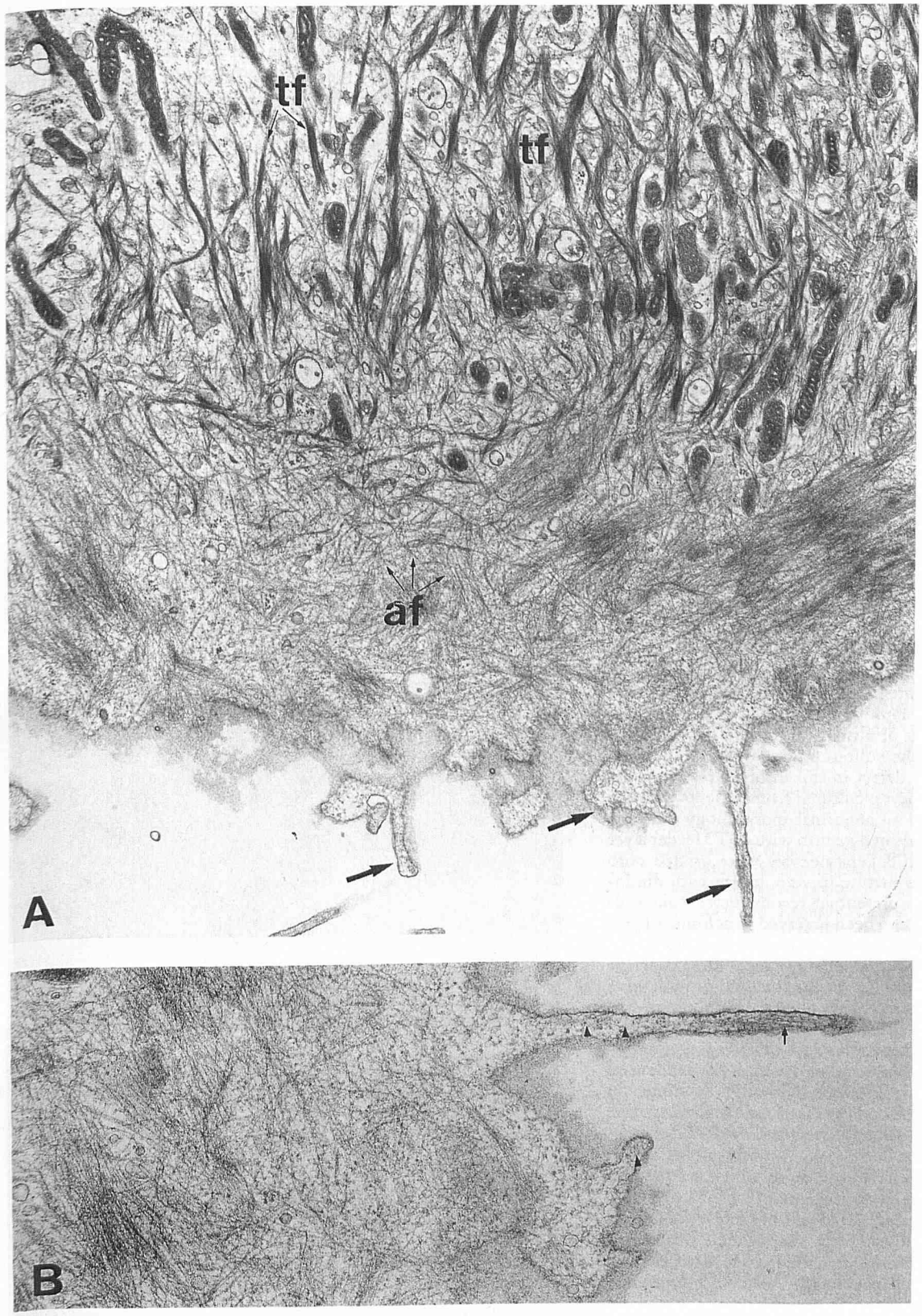

Figure 10. JEB keratinocyte showing details of cytoskeleton and lateral spikes. *(A)* Numerous tonofilaments (tf) are arranged in central area of the cytoplasm, whereas actin filaments (af) predominate in the peripheral cytoplasm. Lateral membrane spikes, some quite poorly formed, are indicated by *arrows* (×14,850). *(B)* Higher magnification of lateral membrane spike, showing lack of well-formed actin filaments, except for a few in extreme terminus *(arrow)*. Less completely formed spikes are completely devoid of actin filaments *(arrowheads)* (×29,700).

ished colony size of JEB keratinocytes might reflect diminished migration of cells in response to calcium or might reflect a lower probability of forming desmosomes or other types of intercellular junctions upon cell-cell contact.

DISCUSSION

Junctional forms of epidermolysis bullosa (JEB) comprise a collection of blistering diseases with defective epidermal adhesion to the basement membrane zone (BMZ), showing separation of the epidermis within the lamina lucida portion of the BMZ in bullous lesions. Many, but not all, cases of JEB are associated with absent, diminished, or altered structure of hemidesmosomes, suggesting that this structure may mediate strong epidermal-dermal cohesion [1–3,27]. The appearance of normal hemidesmosomes in some individuals with JEB suggests the possibility that other mechanisms might also regulate epidermal-dermal adhesion [3]. Furthermore,

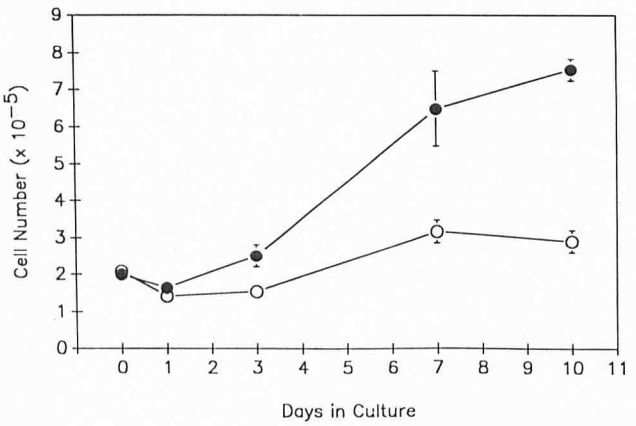

Figure 11. Growth rates of JEB *(open circles)* and normal *(solid circles)* keratinocytes in growth factor-supplemented MDCB 153 medium.

epidermal-dermal cohesion is apparent in fetal development long before formation of hemidesmosomes at 12–16 weeks of gestation, suggesting epidermal-dermal attachment might be mediated independently of hemidesmosomes under some circumstances. Basal keratinocytes in mature epidermis express $\alpha_3\beta_1$ integrin receptors with ligand specificity for laminin, a component of the BMZ [11]. This integrin receptor, which forms part of the actin-filament/focal adhesion complex, could mediate epidermal-dermal attachment independently of the hemidesmosome-anchoring filament-anchoring fibril complex [7–12]. Three antibodies, DEJ-19 [4], AA3 [6], and GB3 [28,29], mark antigens that are often absent in JEB basement membrane zone (BMZ) regions, but these antigens have not yet been mapped to a specific family of cell-adhesion molecules.

Although some component of blister fluid might contribute to extension or propagation of the bullous process in JEB [30], it appears likely that the primary defect in this disorder resides in affected keratinocytes. Keratinocytes cultured from patients with the lethalis form of JEB showed an abnormal morphology with increased birefringence when cultured in vitro with a 3T3 feeder layer [31]. When these cultured JEB keratinocytes were grafted onto mouse dermis and allowed to mature in vivo, bullae with diminished hemidesmosomes were present in reconstructed epidermis [31]. "Blister" formation has also been observed in colonies of JEB keratinocytes that have been allowed to mature in vitro into stratified sheets in high-calcium, serum-containing medium [32]. In the present study, abnormal morphology, adhesion, and growth were observed in JEB keratinocytes cultured in low-calcium, serum-free medium in the absence of extraneous feeder cells and keratinocyte stratification. Differences in adhesion structures were seen in isolated JEB cells or in cell monolayers and thus were not dependent on epidermal stratification or differentiation. Although keratinocytes are artificially removed from a basement membrane in these studies, cultured keratinocytes do synthesize and secrete some BMZ components onto culture surfaces in vitro [10,11], and analysis of cell adhesion in cultured cells permits study of some adhesion structures that are difficult to analyze in intact epidermis.

Two cell-substratum adhesion structures have been identified previously in cultured cells: close contacts that mediate weak adhesions and focal adhesions that mediate strong interactions [7–11]. Focal adhesions, which are probably modified adherence-type junctions, are organization points for the actin cytoskeleton that contain polymerized (f-) actin in addition to α-actinin, talin, vinculin, and specific integrin subtypes [7–16]. In normal cultured keratinocytes, focal adhesions contain both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin subtypes, but strong adhesion to culture substratum (containing endogenous secreted laminin) appears to be mediated specifically via the $\alpha 3\beta 1$ integrin subtype [10,11]. Specific monoclonal antibodies to the $\alpha 3\beta 1$ integrin caused cultured keratinocytes to detach from cultured surfaces [11], whereas a monoclonal anti-$\beta 1$ antibody produced cell-cell detachment and increased cytoplasmic spreading of cultured keratinocytes somewhat similar to appearance of JEB keratinocytes in this study [10].

The defective adhesiveness of JEB keratinocytes examined in this study correlates with abnormal formation of actin-containing focal adhesions. The JEB keratinocyte strain studied here showed diminished "spikes" of polymerized actin at cell margins consistent with diminished focal adhesions which show polymerized actin in this pattern [10,11]. Interference reflection microscopic images and electron microscopic examination also suggest that very close cell-substratum focal adhesions are greatly diminished or absent in these cells. The JEB keratinocytes in culture did display numerous cell-substratum adhesions with the appearance of hemidesmosomes. This was not surprising, because numerous hemidesmosomes were observed in skin biopsy specimens prepared from the same patient. Lamellipodia seen in the ventral membrane surface may result from diminished membrane-substratum adhesiveness and were also identified in electron micrographs of skin biopsies prepared from this patient. Overall, a specific defect in actin filament bundling and formation of actin-containing focal adhesions in this JEB keratinocyte strain is suggested by these data.

The precise molecular defect in formation of focal adhesions has not been identified in these studies. The focal adhesion is a supramolecular complex of numerous cytoskeletal proteins including f-actin, α-actinin, talin, vinculin, and transmembrane α/β integrin receptors, with variable combinations of integrin subunit types possible [7–16]. Assembly of the focal adhesion complex is regulated, in part, by the type and amount of extracellular ligand (matrix) availability [7,9–14]. Assembly and disassembly of the focal adhe-

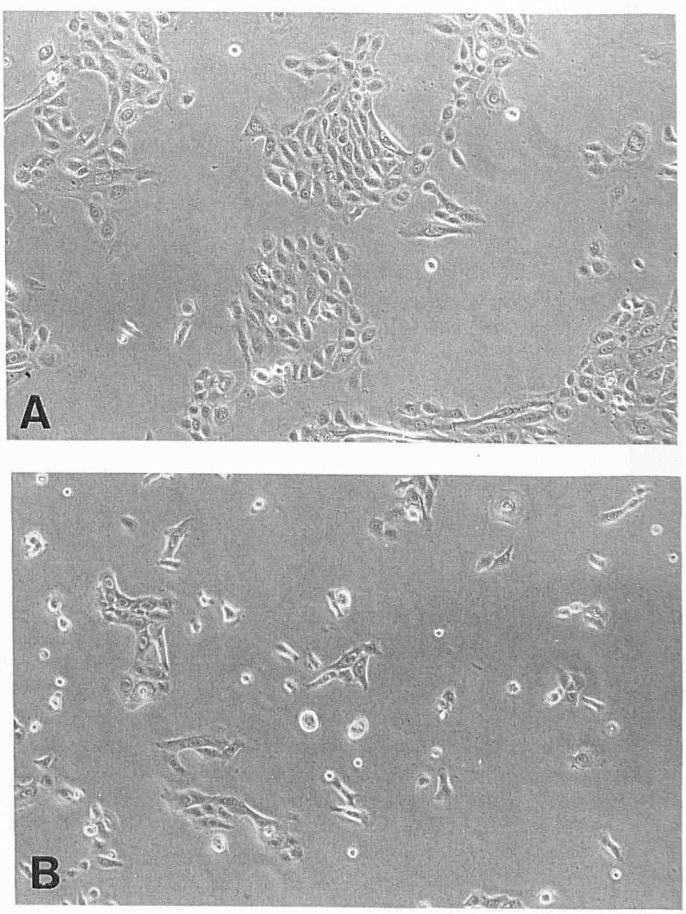

Figure 12. Colony formation by *(A)* normal or *(B)* JEB keratinocytes in medium with high extracellular calcium (1.15 mM). Phase contrast micrographs (×75).

sion complex is also regulated by phosphorylation of vinculin, talin, and integrin proteins on tyrosine residues, and also potentially by interaction of gelsolin with this complex [7,9–14]. Because defective assembly of one protein into this complex could result in its lack of formation, there are multiple molecular targets for potential mutations that could affect assembly of focal adhesions in JEB keratinocytes. The existence of multiple molecular targets might also provide a basis for the multiple clinical phenotypes observed in this disease. However, we need to determine whether long survival of patients with JEB selects for subtypes in which desmosomal attachments are preferentially maintained [3]. Thus JEB keratinocytes from numerous other patients will need to be examined in a similar fashion for defects in the actin filament/focal adhesion complex and for defective formation of hemidesmosomes, which are formed through supramolecular assembly of other, distinct cytoskeletal proteins [7–12].

Finally, the growth characteristics of JEB keratinocytes are markedly altered. Previous work with normal keratinocytes has shown that adhesion to some integrin-containing receptor ligands, e.g., fibronectin or type IV collagen, can increase cell proliferation [33]. Furthermore, laminin (which serves as an attachment ligand for some focal adhesions) has discrete domains with epidermal growth factor homology [34]. Thus, focal adhesions might directly regulate some aspects of cell proliferation via interactions with specific matrix components. Diminished growth, along with the need of focal adhesions for cell migration [7–12], might explain the poor wound healing that is characteristic of patients with this disease. Results of this study suggest that keratinocytes cultured from JEB patients can be used to study and further characterize molecular details of these adhesive- and growth-related cellular functions.

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